Zbl. Bakt. 278, 348–358 (1993)

Analysis of Virulence Factors of Legionella pneumophila

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With 3 Figures

Summary

Legionella pneumophila, the causative agent of Legionnaires' disease is a facultative intracellular bacterium, which in the course of human infection multiplies in lung macrophages predominantly manifesting as pneumonia. The natural habitat of *Legionella* is found in sweet water reservoirs and man-made water systems. Virulent *L. pneumophila* spontaneously convert to an avirulent status at a high frequency. Genetic approaches have led to the identification of various *L. pneumophila* genes. The *mip* (macrophage infectivity potentiator) determinant remains at present the sole established virulence factor. The Mip protein exhibits activity of a peptidyl prolyl *cis trans* isomerase (PPIase), an enzyme which is able to bind the immunosuppressant FK506 and is involved in protein folding. The recently cloned major outer membrane protein (MOMP) could play a role in the uptake of legionellae by macrophages. Cellular models are useful in studying the intracellular replication of legionellae in eukaryotic cells. Human cell lines and protozoan models are appropriate for this purpose. By using U 937 macrophage-like cells and *Acanthamoeba castellanii* as hosts, we could discriminate virulent and avirulent *L. pneumophila* variants since only the virulent strain was capable of intracellular growth at 37 °C. By using these systems we further demonstrated that a hemolytic factor cloned and characterized in our laboratory, legiolysin (lly), had no influence on the intracellular growth of *L. pneumophila*.

Zusammenfassung

Legionella pneumophila, der Erreger der Legionärskrankheit, ist ein fakultativ intrazelluläres Bakterium. Im Zuge einer Infektion der menschlichen Atemwege, die sich als Pneumonie manifestiert, vermehren sich Legionellen intrazellulär in Lungenmakrophagen. Legionellen kommen natürlicherweise in Süßwasserhabitaten, einschließlich Wasserleitungsanlagen vor. Aus virulenten *L. pneumophila*-Stämmen können spontan und mit hoher Frequenz avirulente Varianten entstehen. Genetische Arbeiten führten zur Identifizierung verschiedener *L. pneumophila*-Gene. Bisher konnte nur die *mip* („macrophage infectivity potentiator")-Determinante eindeutig als Virulenzgen identifiziert werden. Das Mip-Protein bildet eine Peptidyl-Prolyl-*cis-trans*-Isomerase (PPIase)-Aktivität aus, die durch Zugabe des

Immunsuppressivums FK506 gehemmt werden kann. Das „major outer membrane protein" (MOMP), dessen genetische Determinante ebenso kürzlich kloniert wurde, ist an der Aufnahme von Legionellen durch Makrophagen beteiligt. Zelluläre Modellsysteme werden zur Untersuchung der intrazellulären Vermehrung von Legionellen in eukaryontischen Zellen herangezogen. Geeignet sind humane Zellinien, aber auch Protozoen-Modelle finden Verwendung. Mit Hilfe der Makrophagen-ähnlichen Zellinie U 937 und *Acanthamoeba castellanii* als Wirtszellen konnten wir virulente von avirulenten *L. pneumophila*-Stämmen unterscheiden, da sich bei 37 °C nur virulente Stämme intrazellulär vermehren. Ferner konnten wir mit diesen Modellen zeigen, daß das Legiolysin (Lly), ein hämolytischer Faktor, der in unserem Labor kloniert und charakterisiert wurde, keinen Einfluß auf die intrazelluläre Vermehrung von *L. pneumophila* nimmt.

Legionella pneumophila, the Causative Agent of Legionnaires' Disease

L. pneumophila was first described in 1977, during the year following the outbreak of epidemic pneumonia in Philadelphia in 1976 (36). The causative agent of the disease, which was termed Legionnaires' disease was identified as a Gram-negative rod-like bacterium with special growth requirements in the laboratory (55). In addition to *L. pneumophila*, 31 species of the genus *Legionella* have been described in the meantime, and half of them have been reported to be pathogenic for humans (13). The clinical manifestation of *Legionella* infections is pneumonia, but also a non-pneumonic disease, termed Pontiac fever, is due to *Legionella* infections.

Legionellae are found ubiquitously in sweet water reservoirs, where the presence of protozoa supports their growth (45). Infection of humans starts with the inhalation of aerosolized water-borne legionellae. Elderly and immunocompromized persons are highly susceptible to infection by legionellae. In the course of infection, which is accompanied by high fever, legionellae multiply in lung macrophages, leading to severe tissue damage (13, 55). Lethal consequences of Legionnaires' disease have been reported quite often, especially in the case of nosocomial outbreaks (41). Transmission from human to human has not been observed.

Legionellae spontaneously convert to an avirulent status, with a relatively high frequency (8). Avirulent variants which can be enriched by culturing on special laboratory media, differ from their virulent parents in numerous aspects. Morphologically, avirulent strains tend to display an enlongated cell shape (39), they furthermore show reduced serum resistance (7) and survival in aerosols (12). Strikingly, the capability to replicate intracellularily in either monocytes and macrophages or even in protozoa is lost (26, 38, 42, 49). Virulence of legionellae is assessed by intranasal or intraperitoneal infection of guinea pigs which seem to have been the only appropriate animal model to date (2, 49). Little is presently known about the factors contributing to the virulence of legionellae. The comparison of virulent and avirulent counterparts is an effective approach which results in a better understanding of *Legionella* pathogenicity. In addition to this approach, genetic attempts to clone and characterize putative *Legionella*-specific virulence factors have been undertaken in recent years.

The Mip Protein: A Legionella Virulence Factor with an Unusual Enzymatic Activity

Several groups have reported about the construction of *Legionella* genomic libraries from patient isolates (17, 20, 21). Various *L. pneumophila*-specific proteins have been cloned in *E. coli* K-12. The most intensively studied protein is the so-called Mip ("macrophage infec-

tivity potentiator") factor, a membrane-associated protein of 24 kDa (15, 17). It was conclusively demonstrated that the ability of Mip-negative mutants of *L. pneumophila* to invade eukaryotic cells was reduced by a factor of 100 when compared to Mip-positive cells (11). In addition, a mutation in the *mip* gene resulted in an attenuation of virulence as estimated in the guinea pig model (10). *In vivo* and *in vitro* virulence could be restored by reintroduction of functionally active *mip* sequences into the Mip-negative mutants.

The *mip* sequences are highly conserved among virulent and non-virulent strains of *L. pneumophila* (Table 1, ref. 9). It exhibits homology with *mip*-like genes of other *Legionella* species (1, 9). Interestingly, genes which show a degree of about 50% homology to the *mip* determinant of *L. pneumophila* are also located on the genomes of other pathogenic bacteria like *Chlamydia trachomatis, Neisseria meningitidis* and *Pseudomonas aeruginosa*, thus furthering the argument of a common mechanism for these proteins in the pathogenesis of bacteria.

The amino acid sequence from the C-terminal part of the Mip protein also shares homologies with eukaryotic FK506 binding proteins (FKBPs) which, together with ciclophilins, form the substance class of immunophilins. They are involved in the regulation of early T-cell activation (47). The immunophilins are able to bind the immunosuppressants, FK506 (in the case of FKBPs) and ciclosporin (in the case of ciclophilins). In addition, they exhibit peptidly prolyl *cis trans* isomerase (PPIase), activity, i. e. they isomerize *cis* peptidyl-proline bonds to the *trans* configuration and may play a role in protein folding (19, 30). Consequently, we expressed the Mip factor in a vector system suitable for the isolation and characterization of the protein. To our surprise, we were able to show that Mip indeed

Table 1. Homology between the amino acid sequence of the Mip protein of *L. pneumophila* strain Philadelphia I (data from ref. 18) and sequences and open reading frames (ORFs) of various organisms

Organism	Protein	Homology	References
Legionella pneumophila strain Wadsworth	Mip	99.6%	16
Legionella pneumophila strain U21 S6	Mip	99.6%	*Ludwig*, unpublished data
Legionella micdadei	Mip-like protein	88.4%	1
Neisseria meningitidis	ORF in the vicinity of pili genes	59.8%	43
Chlamydia trachomatis	L2-Protein[1]	56,2%	32
Pseudomonas aeruginosa	ORF in the vicinity of alginate synthesis genes	50,0%	28
Man	FKPB of T cells[2]	55,2%	33, 48
Saccharomyces cerevisiae	FKBP	54%	54
Man	FKBP of human cancer cell line	53,4%	27
Neurospora crassa	FKBP	50,8%	53

[1] The sequence of th C-terminal part of the L2 protein was used for comparison.
[2] FK506 binding protein.

exhibited PPIase activity which could be inhibited by FK506 in a nanomolar range (18). Presently studies are underway to decide whether or not this particular enzymatic activity contributes to the pathogenicity of *Legionella*.

Molecular Cloning of Other Putative Virulence Factors

Besides the Mip protein, several other *Legionella* antigens were identified and characterized which may function as virulence factors (see Table 2). A recently done by *Engleberg* and others (16) the 19 kDa peptidoglycan-associated protein of *Legionella* (Ppl) has been cloned and sequenced in our laboratory (31). This protein is associated with the murein layer of the bacterial surface and exhibits a distinct homology to lipoproteins of *E. coli* and *Haemophilus influenzae*. The role of Ppl in pathogenicity of legionellae has to be established yet.

The major outer membrane protein (MOMP) of *L. pneumophila* has been previously cloned (24). The MOMP protein binds the C3 factor of the complement, thereby mediating the macrophageal engulfment of *Legionella* (3). The gene *omp*S encodes the 28 kDa protein which also can be covalenty linked to peptidoglycan, forming a structure of 31 kDa. Oligomers of 28 kDa and 31 kDa form a complex of 100 kDa which can be isolated from *Legionella* surfaces (24, 25). The MOMP complex is further

Table 2. *L. pneumophila* genes cloned in *E. coli* K-12

Gene	Mol. weight of the protein	Function/Description	Contribution to pathogenicity	References
mip	24 kDa	"macrophage infectivity potentiator", involved in intracellular survival, PPIase activity	+	10, 11, 18
msp	38 kDa	"major secretory protein" metallo-protease, cytotoxic and hemolytic activity	–	5, 29, 51
lly	39 kDa	"legiolysin", hemolytic activity, involved in pigment production	–	56
*ppl*A	19 kDa	"peptidoglycan-associated protein of *Legionella*"	n.d.	16, 31
*omp*S	28 kDa–31 kDa	Major outer membrane protein (MOMP), 100 kDa complex, involved in uptake of legionellae by C3-opsonization, attachment	+	3, 24, 25
*htp*B	60 kDa	"Heat shock proteins", housekeeping functions	n.d.	22, 23, 46
*sod*B	n.d.	Iron-superoxide dismutase	n.d.	50
*rec*A	38 kDa	homologous recombination UV-induced	n.d.	57

n.d., not determined.

involved in an opsonization-independent attachment to human cells (44). Furthermore, the 60 kDa heat shock protein (Htp) of *L. pneumophila* was cloned by *Hoffman* et al. and other groups (20, 22, 46). It is immunodominant, displaying homology to the *gro*EL family of heat shock proteins (23). Mutations in the *htp* locus indicating that the "house-keeping" functions of this protein would make it indispensable for the bacterium could not be obtained.

Two hemolytic proteins have been cloned, and one of them, the Msp (major secretory protein) is 38 kDa metallo-protease for which also cytotoxic effects on CHO cells have been demonstrated (5, 29). On the other hand, Msp-negative mutants remain virulent (51). The other hemolytic protein, legiolysin (Lly), was intensively studied in our laboratory. The 39 kDa protein expressed in *E. coli* K-12 exhibited brown pigment production in addition to hemolysis (56). Recently, an iron-superoxide dismutase which may be involved in the defence against the intracellular attack has been cloned from *L. pneumophila* (50). Additionally, the 38 kDa RecA homologous protein of *L. pneumophila* was cloned (57) and shown to have functions similar to the *E. coli* RecA protein.

The cloned genes of *L. pneumophila* were also useful for differentiation of *L. pneumophila* strains from isolates of other species. DNA probes derived from the legiolysin *(lly)* and the peptidoglycan-associated protein of *Legionella (ppl)* were used in the study of a large number of strains. We found that under conditions of high stringency hybridization, *L. pneumophila* reacted exclusively with these DNA probes. By applying low stringency conditions for hybridization, the genus *Legionella* could be differentiated from other Gram-negative bacteria (4, 40).

Construction of Genomic Mutants

Genetic attempts to analyse putative virulence factors of legionellae were hindered by numerous obstacles. Legionellae harbor a restriction system which functions as a defense against incoming DNA (35). Heterologous transfer of plasmids can only be achieved *via* conjugation and at relatively low frequency. Counterselection necessary for the isolation of *trans*-conjugants is restricted to a narrow spectrum of antibiotic resistance (37). To date, random transposon mutagenesis did not lead to an identification of virulence factors with the result that factors other than those obtained in the *E. coli* K-12 system (see above) have not been identified genetically. Genomic mutations were created by allelic exchange mutagenesis using the cloned genes. One of the approaches had been the use of mobilizable *pir*-dependent plasmids from the *inc*X group (51). These were shown to be transferred to *Legionella*. They do not replicate due to the lack of the Pir protein in the recipient. Mutations can be therefore obtained by selecting the recombinational event concomitantly with the introduction of an antibiotic resistance marker. Experiments utilizing Col E1-derived plasmids for mutagenesis *via* allelic exchange have also been succesfully reported (11).

We inserted a kanamycin resistance gene into the legiolysin-coding sequence. The respective fragment was subcloned into pMSS 704-1. This plasmid is a derivative of the *pir*-dependent R6K (52) carrying an additional chloramphenicol resistance marker. Heterospecific conjugation was carried out between the pir^+ *E. coli* donor in which the genetic manipulations had been performed and a restriction-deficient derivative of the *L. pneumophila* Philadelphia I strain, JR 32 (35). Mobilization of the construct was achieved through the chromosomal *tra* function of the *E. coli* donor which acted upon

the *mob* sequences present on the plasmid. Trans-conjugants were selected by streptomycin resistance (for the recipient *L. pneumophila* JR 32) and kanamycin resistance for allelic exchange of the wild type *lly* locus with the mutant DNA. Integration of the plasmid was excluded by the chloramphenicol-sensitive phenotype of the trans-conjugants (Fig. 1).

The mutants obtained were characterized genetically and phenotypically showing that disruption of the *lly* gene by the insertion of kanamycin resistance resulted in a loss of brown pigment production. Interestingly, a marked influence on the hemolytic activity of the mutant could not be observed. This has been explained by the fact that *L. pneumphila* carries additional hemolytic factors which may mask the hemolytic activity of the legiolysin (6). Reintroduction of intact *lly* sequences by transferring them via a stable replicating pMMB 34 derivative (*inc*Q) resulted in restoration of brown pigment production. The influence of the *lly* mutation was also tested in models for intracellular replication of *Legionella*.

Cellular Models for Intracellular Replication

Various cellular models for intracellular replication of *Legionella* have been established. It could be shown that human cell lines are appropriate for this purpose. These include HeLa cells (14), Hl 60 monocytes (34) and U 937 macrophage-like cells (42). In addition, recent data have shown that protozoan models such as *Acanthamoeba castellanii* are especially useful in the differentiation of virulent from avirulent variants (38,

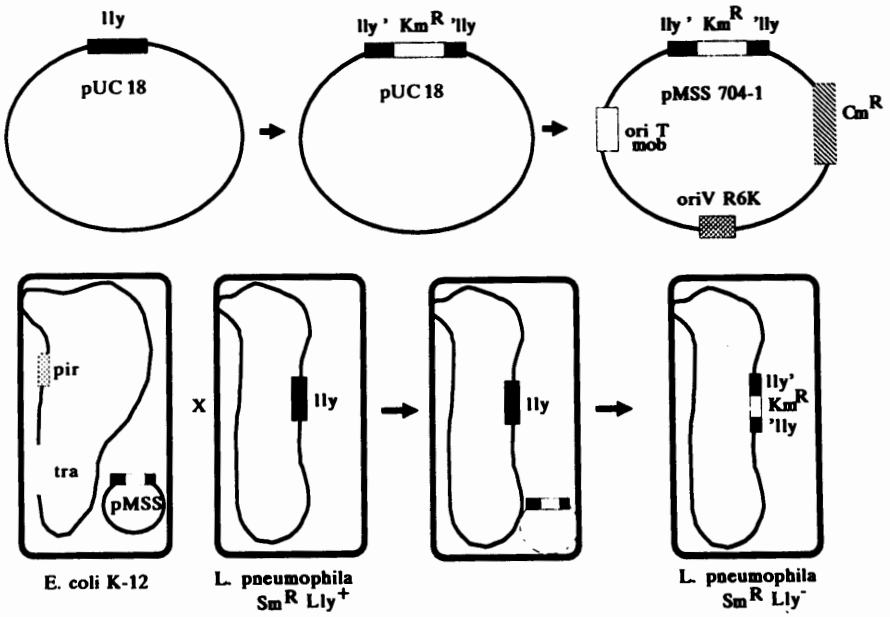

Fig. 1. Construction of a Lly^- mutant of *L. pneumophila* Philadelphia I (JR 32) by allelic exchange.

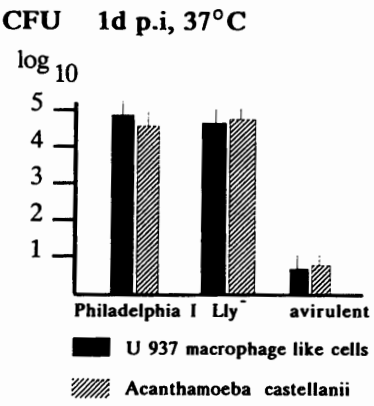

Fig. 2. Intracellular growth behavior of *L. pneumophila* Philadelphia I, an avirulent derivative (XXXV), and the Lly⁻ mutant in U937 macrophage-like cells and *A. castellanii* grown at 37 °C. Mean CFU values (with standard deviation) determined 1 d after infection are given.

49). We tested the Lly-negative *L. pneumophila* mutant for intracellular growth in U 937 macrophage-like cells and in *A. castellanii*. The invasion assay begins with the centrifugation of the legionellae onto the host cells and an incubation for 2 hours, so that legionellae can establish themselves intracellularily. Hereafter, the supernatant is removed and remaining extracellular legionellae are killed by gentamicin treatment (80 µg/ml) applied for one hour. After washing, fresh antibiotic free-medium is added. Colony-forming units of viable legionellae are counted at time "O", i. e. directly after gentamicin treatment, 3 and 1 day after infection (p. i.), by plating serial dilutions of the disrupted cultures on special *Legionella* medium.

The tests were performed using the virulent Philadelphia I strain, an avirulent derivative (XXXV) and the Lly-negative mutant at 37 °C. In Fig. 2 it can be seen that 1 d p. i., the Lly-negative mutant "displayed intracellular CFU values" similar to those obtained for the virulent Philadelphia parent. From these data, we conclude that the legiolysin has no influence on intracellular replication of *L. pneumophila*. Perhaps the involvement of *lly* in pigment production confers an advantage to legionellae with regard to their survival in the environmental reservoir. Fig. 3 illustrates the intracellular location of legionellae in phagosomes of U 937 cells and, during the later stage of the intracellular life cycle, in the cytoplasm. Since the growth temperature had been reported to have an influence on the survival of *L. pneumophila* in *A. castellanii* (38), the strains were further tested at 30 °C with *A. castellanii* as a host (Table 3). It could be shown that the avirulent derivative of the Philadelphia I strain was also capable of growth in *Acanthamoeba* at 30 °C (49).

Acknowledgements. Our own studies included in this work were supported by the Bundesministerium für Forschung und Technologie (BMFT 01 K; 8829). We gratefully acknowledge the critical comments of *Laurence R. E. Phillips* and the editorial assistance of *H. Kurz*.

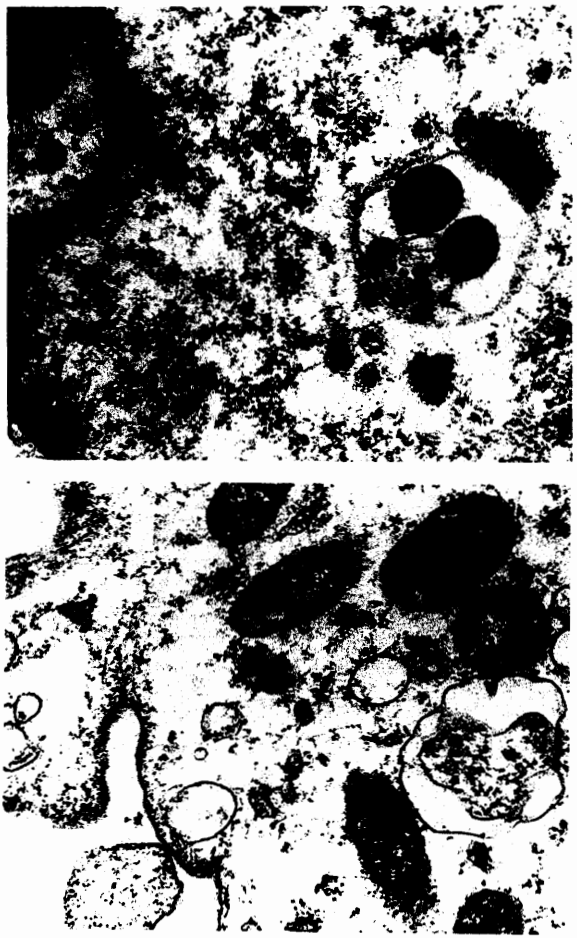

Fig. 3. Electronmicroscopic examination of thin sections of U937 cells infected by legionellae. The intracellular location of *L. pneumophila* in phagosomes (above) in the early stage of invasion is shown (magnification 20 000×). In a later stage of the intracellular life cycle, legionellae are found in the cytoplasm (below, magnification 30 000×).

Table 3. Intracellular growth behavior and *in vivo* virulence of *L. pneumophila* Philadelphia I and derivatives

Strain	Intracellular growth in U 937 cells	*A. castellanii*		*in vivo* virulence (i.p. infection of guinea pigs)
		30 °C	37 °C	
L. pneumophila Philadelphia I	+	+	+	+
L. pneumophila XXXV, avirulent	−	+	−	−
L. pneumophila JR 32, Lly^-	+	+	+	n.d.

n.d., not determined.

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